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# Photoinduced Affinity Labeling of the *Escherichia coli* Ribosome Puromycin Site†

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ABSTRACT: The photoincorporation of puromycin into *Escherichia coli* ribosomes has been studied in detail. Incorporation into protein L23 as a function of puromycin concentration follows a simple saturation curve and is specifically blocked by structural and functional analogues of puromycin, thus demonstrating that such incorporation proceeds via an affinity labeling process. Incorporation into L23 becomes more specific as the light fluence is reduced, indicating that such incorporation takes place from a native rather than light-denatured puromycin site. L23 remains the major labeled protein using ribosomes prepared by several procedures, suggesting the conservative nature of the site. In addition evidence is presented for affinity labeling of S14 and of a site in the RNA fraction of the 50S particle. Specific incorporation appears to proceed with an anomalously high quantum yield. The detailed photochemical mechanism is not understood, although 8-alkylation of the purine moiety has been excluded. Incorporation is largely inhibited in the presence of thiol reagents.

Affinity labeling has emerged as a powerful tool for exploring ligand–receptor interactions (Knowles, 1972; Singer, 1967; Cooperman, 1976). This is especially true for the *Escherichia coli* ribosome, numerous articles having appeared recently on affinity labeling studies between this particle and reactive derivatives of tRNA, mRNA, antibiotics, and guanine nucleotides.[1] We have previously shown (Cooperman et al., 1975) that on ultraviolet (UV) irradiation both puromycin and *N*-ethyl-2-diazomalonylpuromycin incorporate into *E. coli* ribosomes, although at what appear to be distinct sites. In the present paper we present strong evidence, based on saturation and analogue competition studies, for what was previously only a suggestion, that the photoincorporation of puromycin into the ribosomal protein L23 proceeds by affinity labeling. We also present evidence suggesting the concurrent affinity labeling of S14 as well as of an as yet unidentified site in the RNA fraction of the 50S subunit.

In several other affinity labeling studies on ribosomes, different investigators using similar or identical reagents have reported apparently contradictory results (Pellegrini et al., 1974; Breitmeyer and Noller, 1976; Yukioka et al., 1977; Pongs et al., 1973; Sonenberg et al., 1973, 1975; Hsiung and Cantor, 1974; Hsiung et al., 1974; Girshovich et al., 1974), and conclusive explanations have not been put forward to account for the divergencies. These results may not be surprising in view of the complexity and conformational mobility of the *E. coli* ribosome (Spitnik-Elson and Elson, 1976; Guinzburg and Zamir, 1975; Petersen et al., 1976 Barbacid and Vazquez, 1975), the quite different methods commonly used in its purification, and the variety of reaction conditions used in labeling experiments. They do, however, emphasize the need for exploring the labeling pattern obtained as a function of several variables in order to avoid obtaining results reflecting procedural artifacts. In this paper we report studies on the photoincorporation of puromycin into ribosomes upon variation of puromycin concentration, light fluence, ribosomal preparation, salt concentration, and irradiation source.

## Experimental Section

Buffers used included: (A) 10 mM Tris-HCl (pH 7.8), 10 mM $Mg(OAc)_2$, 50 mM KCl; (B) 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM KCl; (C) 50 mM Tris-HCl (pH 7.4), 0.3 mM $MgCl_2$, 50 mM KCl; (D) 100 mM Tris-HCl (pH 7.4), 12 mM $MgCl_2$, 100 mM $NH_4Cl$, 6 mM 2-mercaptoethanol.

Scintillation cocktails used were: (I) 3:1 toluene (0.5% PPO,[2] 0.01% $Me_2POPOP$)–Triton X-100; (II) toluene (0.5% PPO, 0.01% $Me_2POPOP$).

*Materials.* Puromycin dihydrochloride and puromycin

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[1] This subject material has been reviewed recently (Cooperman, 1978; Cantor et al., 1974; Pongs et al., 1974).

[2] Abbreviations used are: PANS, puromycin aminonucleoside; Phe-PANS, *N*-phenylalanylpuromycin aminonucleoside; CpA, cytidylyl-(3′–5′)-adenosine; CAPhe, cytidylyl-(3′–5′)-3′-*O*-phenylalanyladenosine; AcPhe, *N*-acetylphenylalanine; PPO, 2,5-diphenyloxazole; $Me_2POPOP$, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; Temed, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

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aminonucleoside (PANS) were purchased from Sigma. [$^3$H]Puromycin (3820 Ci/mol) labeled in the methoxy protons, [$CH_3$-$^3$H]puromycin, and [$^{14}$C]phenylalanine (444 Ci/mol) were purchased from New England Nuclear. *N*-Phenylalanylpuromycin aminonucleoside (PhePANS) was prepared as described (Harris et al., 1972). [$^3$H]Puromycin and [$^3$H]-PhePANS (1.5 Ci/mol) labeled in the H-8 position, [8-$^3$H]-puromycin and [8-$^3$H]PhePANS, were prepared by exchange with tritiated water, as described (Shelton and Clark, 1967). [8-$^3$H]Puromycin (3500 Ci/mol) was also purchased from Amersham/Searle. Cytidylyl-(3′–5′)-3′-*O*-phenylalanyl-adenosine (CAPhe) (Chladek et al., 1974) was the generous gift of Dr. S. Chaldek, Cytidylyl-(3′–5′)-adenosine (CpA) was obtained on hydrolysis of CAPhe. Purified EF-Tu was the generous gift of Dr. D. L. Miller (Miller and Weissbach, 1974). Purified L23 was the generous gift of Dr. M. Nomura. Polysomes (denoted as P) derived from *E. coli* MRE 600 were the generous gift of Dr. S. Pestka (Pestka and Hintikka, 1971). Phe-tRNA$^{Phe}$ was prepared as described (Ravel and Shorey, 1971) using bulk stripped tRNA (Grand Island Biological) and crude factors from ribosomal preparations as a source of synthetase. AcPhe-tRNA$^{Phe}$ was prepared and purified on Sephadex G-25 according to Haenni and Chapeville (1966), giving 36 ± 1 pmol of AcPhe per $A_{260}$ unit of bulk tRNA. Acylation of phenylalanine was shown to be quantitative by thin-layer chromatography (TLC) analysis of a sample subjected to mild base hydrolysis.

*Preparation of Ribosomes.* All ribosomes were prepared from *E. coli* Q13 bacteria harvested in late log phase. Three methods were used. The first was that of Kurland (1966), giving ribosomes denoted K. The second was that of Traub et al. (1971), giving ribosomes denoted as T, except that the S-30 is treated with 0.1 mM puromycin for 30 min at 37 °C prior to preparation of S-100 pellet and that the S-100 pellet is resuspended in a buffer containing 15 mM $Mg^{2+}$ (Noll et al., 1973). In the third method, deoxycholate-washed ribosomes (denoted as D) were prepared by resuspending an S-100 ribosomal pellet in buffer A containing 6 mM 2-mercaptoethanol and 0.05% deoxycholate, spinning the solution in a SS34 rotor for 10 min at 10 000 rpm, removing the supernatant, and re-pelleting the ribosomes by centrifugation in a Ti50 rotor for 3 h at 50 000 rpm. Unless otherwise noted, all steps in ribosomal preparation were conducted at 4 °C. Redissolved ribosomal pellets were stored in 0.1-mL aliquots at −78 °C at concentrations greater than 400 $A_{260}$ units/mL.

*Photolytic Incorporation of Puromycin.* Photolysis experiments were performed as previously described (Cooperman et al., 1975) using three different sets of Rayonet lamps, RPR-2537Å, RPR-3000Å, and RPR-3500Å (Southern New England Ultraviolet Co.). A Pyrex filter having 0.5% transmittance below 280 nm was used with the latter two lamps. Potassium ferrioxalate actinometry (Hatchard and Parker, 1956) showed the incident dose to be $7 \times 10^{16}$ quanta/(s mL) with the 2537-Å lamps and $11 \times 10^{16}$ quanta/(s mL) with the 3500-Å lamps. Unless otherwise stated all photolysis experiments were performed in buffer B. Thawed K ribosomes were heat activated at 37 °C for 60 min prior to use. Thawed T ribosomes were used directly. Unless otherwise noted, following photolysis puromycin-labeled ribosomes were removed from free puromycin by repeated ethanol precipitation (Cooperman et al., 1975). Puromycin incorporation into 70S particles and into the RNA and protein fractions was determined as described previously, using the same procedures for background (defined as incorporation measured in the absence of irradiation) and internal absorbance corrections (Cooperman et al., 1975). Ethanol pellets were resuspended in buffer C and separated into 30S and 50S subunits by centrifugation through a 5–20% sucrose gradient in buffer C (SW50.1 rotor, 45 000 rpm, 135 min), and the gradients were fractionated using a Beckman flow cell. Fractions containing subunits were pooled, the $Mg^{2+}$ concentration was adjusted to 10 mM, and subunits were precipitated by addition of 2 vol of 95% ethanol. Protein was extracted using the $Mg^{2+}$–acetic acid procedure (Hardy et al., 1969) and puromycin incorporation into the protein and RNA fractions of each particle was determined as described (Cooperman et al., 1975).

Liquid scintillation counting of total ribosome and ribosomal RNA was performed by dissolving the material in 0.25 N NaOH and adding to 0.6 mL of the basic solution 0.1 mL of glacial acetic acid and 7 mL of scintillation cocktail I. Counting of protein extracts was performed by combining an aliquot of the 67% acetic acid extract in a total aqueous volume of 0.6 mL with 7 mL of scintillation cocktail I.

*Polyacrylamide Gel Electrophoresis.* One-dimensional gel electrophoresis on extracted and lyophilized proteins was performed using a slab apparatus (Studier, 1973) as described by Leboy et al. (1964) (10% acrylamide, 0.15% bis(acrylamide)) except that the separation gel buffer concentration was half that described by Reisfeld et al. (1962). Proteins were visualized as described by Howard and Traut (1973). Gel strips were frozen in dry ice and sliced into approximately 2-mm slices (Matsumura and Noda, 1973), which were dried in shell vials (at room temperature or 50 °C) and digested with 0.35 mL of a 30% hydrogen peroxide–concentrated $NH_4OH$ (99:1) solution by incubation for 5 h at 50 °C (Goodman and Matzura, 1971). The vials were then allowed to stand overnight at room temperature, 4.0 mL of scintillation cocktail I was added to each vial, and the samples were counted. The recovery of radioactivity was determined by summing across the gel and correcting for counting efficiency. When over 1500 cpm per sample was applied, the recovery from gel to gel was 50 ± 10%, but samples from a given slab gel showed smaller variation, ±3% for 50S proteins and ±6% for 30S proteins. Values of incorporation listed in Table I were obtained by correcting the counts observed in a particular gel region for the total loss of radioactivity on the gel, and assuming that 1 $A_{260}$ unit corresponds to 26 pmol of 70S ribosomes, 39 pmol of 50S ribosomes, and 78 pmol of 30S ribosomes.

Two-dimensional gel electrophoresis was performed as described by Kaltschmidt and Wittmann (1970). Staining and determination of radioactivity in small gel areas were conducted essentially as described above, except that in some cases gel was oxidized in a Packard sample oxidizer and radioactivity was determined as tritiated water.

*Assay of AcPhe Binding and Transfer to Puromycin.* [$^{14}$C]AcPhe-tRNA binding to ribosomes (Duquette et al., 1974) was determined by incubating [$^{14}$C]AcPhe-tRNA and ribosomes in buffer D containing 1.2 mM GTP and 0.12 mg/mL poly(U) (Miles) for 10–12 min at 28 °C, filtering an aliquot onto a type HAWP 0.45 μm Millipore filter, adding 3.5 mL of scintillation cocktail II to the dried filter and counting. Binding found in the absence of poly(U) was about 6% of that found in its presence. To measure transfer from bound [$^{14}$C]AcPhe-tRNA to puromycin, the binding reaction mixture described above was allowed to incubate for 30 min at 28 °C and puromycin was added to a final concentration of 0.5 mM. After an additional 30-min incubation at 28 °C, an aliquot was removed and mixed with saturated $MgSO_4$ in 0.1 M potassium acetate (pH 5.7) (Monro, 1971). Extraction with ethyl acetate and subsequent liquid scintillation counting of the ethyl acetate layer afforded a measure of AcPhe transfer to puromycin. The presence of EF-Tu (30 μM) in the incu-

TABLE I: Distribution of Photoincorporated Label in One-Dimensional Gels.

| | | | | | | 50S protein gels | | 70S protein gels | | |
|---|---|---|---|---|---|---|---|---|---|---|
| No. | Ribosomal prepn | [KCl] (mM) | [Puro] (mM) | Lamp | Photolysis time (min) | Region IV puro/50S (%) | Region II normalized[a] | Region IV puro/70S (%) | Region II normalized[a] | Region VI normalized[a] |
| 1 | K | 50 | 0.078 | 2537 | 2 | 0.13 | 0.55 | | | |
| 2 | K | 50 | 0.078 | 2537 | 8 | 0.69 | 0.73 | 1.05 | 1.17 | 0.89 |
| 3 | K | 50 | 0.039 | 2537 | 16 | 1.02 | 0.90 | | | |
| 4 | P | 50 | 0.156 | 2537 | 8 | 2.45 | 0.91 | | | |
| 5 | D | 50 | 0.078 | 2537 | 8 | | | 1.07 | 1.13 | 0.41 |
| 6 | T | 50 | 0.044 | 2537 | 8 | | | 0.94 | 0.85 | 0.55 |
| 7 | T | 50 | 0.074 | 2537 | 8 | | | 1.23 | 0.63 | 0.30 |
| 8 | T | 50 | 0.140 | 2537 | 8 | 0.63 | 0.55 | | | |
| 9 | K | 50 | 0.078 | 3000 | 8 | | | 0.51 | 1.57 | 1.03 |
| 10 | T | 50 | 0.044 | 3500 | 8 | | | 0.40 | 0.72 | 0.52 |
| 11 | T | 50 | 0.131 | 3500 | 20 | | | 1.62 | 0.75 | 0.56 |
| 12 | T | 25 | 0.30 | 3500 | 30 | | | 3.6 | 0.84 | 0.49 |
| 13 | T | 55 | 0.30 | 3500 | 30 | | | 3.5 | 0.66 | 0.44 |
| 14 | T | 105 | 0.30 | 3500 | 30 | | | 2.7 | 0.63 | 0.36 |
| 15 | T | 205 | 0.30 | 3500 | 30 | | | 4.2 | 0.57 | 0.39 |
| 16[b] | K | 50 | 0.070 | 2537 | 8 | 0.30 | 1.27 | | | |
| 17[c] | K | 50 | 0.074 | 2537 | 8 | | | 0.56 | 1.91 | 0.88 |
| 18 | T | 50 | 0.150 | 3500 | 120 | | | 5.94 | 1.18 | 0.97 |
| 19[d] | T | 50 | 0.150 | 3500 | 120 | | | 2.42 | 2.34 | 1.20 |

[a] Radioactivity in region IV set equal to 1.00. [b] In the presence of 0.165 mM CAPhe. [c] In the presence of 0.245 mM CAPhe. [d] In the presence of 2.1 mM PhePANS.

bation mixtures was found to increase AcPhe-tRNA binding approximately twofold (from 0.2/ribosome to 0.4/ribosome) with a proportionate increase in AcPhe transfer to puromycin.

To measure transfer from bound [$^{14}$C]AcPhe-tRNA to covalently incorporated puromycin, ribosomes were photolyzed in the presence of [$CH_3$-$^3$H]puromycin at 3500 Å and then dialyzed against buffer D at 5 ± 1 °C until the concentration of puromycin in the dialysis bag fell below 0.1 μM. Ribosomes thus prepared were added to the binding mixtures as described above and aliquots were assayed for [$^{14}$C]AcPhe-tRNA binding and for [$^{14}$C]AcPhe transfer to added, nonradioactive puromycin as described above. The bulk of the sample was treated with 0.5 mM puromycin to release most of the noncovalently bound AcPhe and the ribosomes were precipitated by addition of two parts of ethanol. The resulting ethanol pellet was dissolved in buffer C and resolved into charged tRNA, 30S, and 50S fractions by ultracentrifugation as described above. Dual isotope counting methods were used to estimate moles of [$^{14}$C]AcPhe and moles of [$CH_3$-$^3$H]puromycin incorporated per mole of 50S subunit. Alternatively, the ethanol pellet was extracted with acetic acid and the radioactivity in the protein fraction was determined.

## Results

*Puromycin Incorporation into Ribosomal Proteins.* We have previously reported that two-dimensional gel electrophoretic analysis of proteins extracted from 70S ribosomes labeled with puromycin showed a single region of the gel labeled to a large extent above background (Cooperman et al., 1975). We now present these results in detail. Figure 1 shows the results of three different gel electrophoretic analyses of labeled ribosomal proteins, in which the radioactivity in the region of interest is localized at progressively higher resolution. These results place the radioactive peak as partially overlapping with protein L23 but centered to the upper left of it. One-dimensional gel electrophoretic analysis of total 50S and 70S proteins (Figures 2 and 3) shows one large peak (region IV) whose mobility is just slightly retarded with respect to authentic L23 (one gel slice or 0.015 $R_f$ unit), corresponding to the large peak seen in the two-dimensional gels. A one-dimensional gel of total 30S protein (Figure 4) shows two major radioactive peaks, neither of which overlaps with the major radioactive peak seen in Figures 2 and 3.[3]

A comparison of two-dimensional gel electrophoresis results at high (2 mM) and low (0.1 mM) puromycin concentrations, with a constant amount of puromycin radioactivity, is presented in Figure 5. High puromycin strongly inhibits incorporation into the major radioactive peak, as well as into the second highest peak, which, using an approach analogous to that described in Figure 1, we have shown to comigrate with S14. No clearly significant differential labeling is found for any other protein.

A one-dimensional gel analysis of the labeling pattern of 50S protein as a function of puromycin concentration is presented in Figure 2. As with the two-dimensional gel experiment, the total radioactivity in each labeling mixture remains constant, so the specific activity decreases as puromycin concentration increases. A plot of the amount of puromycin incorporated into region IV as a function of puromycin concentration shows simple saturation behavior (Figure 6), giving, in two independent experiments, dissociation constants of 0.62 ± 0.28 mM (curve a) and 0.78 ± 0.12 mM (curve b), for an average value of 0.70 ± 0.20 mM. Similar plots of the other regions of the gels containing significant amounts of radioactivity, region II in Figure 2 (50S protein) and regions II′ and VI′ in Figure 4 (30S protein), showed incorporation to increase linearly as a function of puromycin concentration (Figure 7), indicating no saturation of incorporation.

[3] In Figures 2–4, gel slices 1–5 correspond to the stacking gel. Radioactivity in this region was quite variable from sample to sample. The large peak of radioactivity in region VII of Figure 3, which does not correspond to protein, is found on gel electrophoresis of proteins extracted from 70S ribosomes, but not on gel electrophoresis of proteins extracted from the 30S and 50S subunits. This result and other evidence leads us to conclude that this radioactivity represents incorporation into a low molecular weight component of 70S ribosomes which is removed following dissociation of the 70S particle and isolation of the subunits.

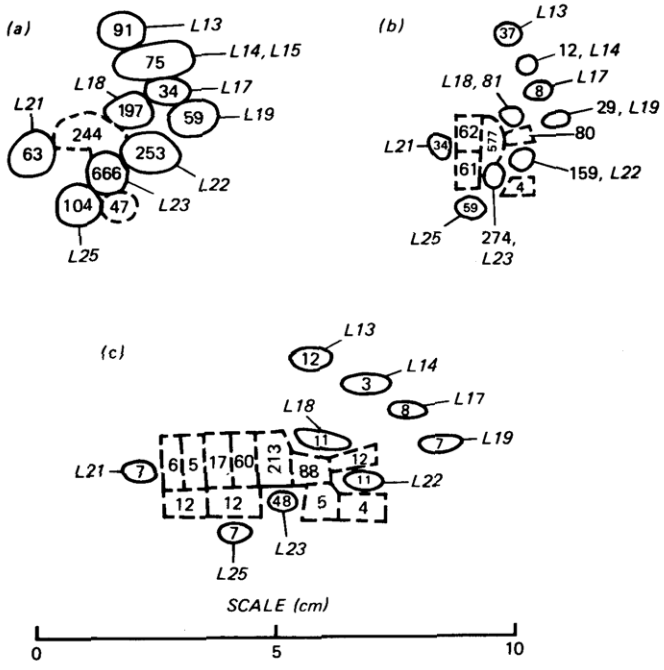


FIGURE 1: Region surrounding the major radioactive peak on two-dimensional gel electrophoresis of labeled ribosomal proteins. Solid lines surround areas of protein staining. Block numbers indicate counts per minute found in each gel area. Numbers are not comparable from one gel to another, since different size samples were used. In gel (a) large areas around each protein were cut out and the radioactivity contained therein determined, thus locating major areas of radioactivity. In gel (b), areas were cut for each protein corresponding closely to the observed staining. In gel (c), sample was applied to the anodic end, rather than in the middle, of the first dimension, thus increasing the resolution in the region of interest. Protein areas were cut as in (b). Experimental conditions: 104 $A_{260}$ units/mL K ribosomes; 0.013 mM [$CH_3$-$^3$H]puromycin; photolysis was for 8 min at 2537 Å. Essentially identical results were obtained when T ribosomes were photolyzed in the presence of [8-$^3$H]puromycin using the 3500-Å lamps.

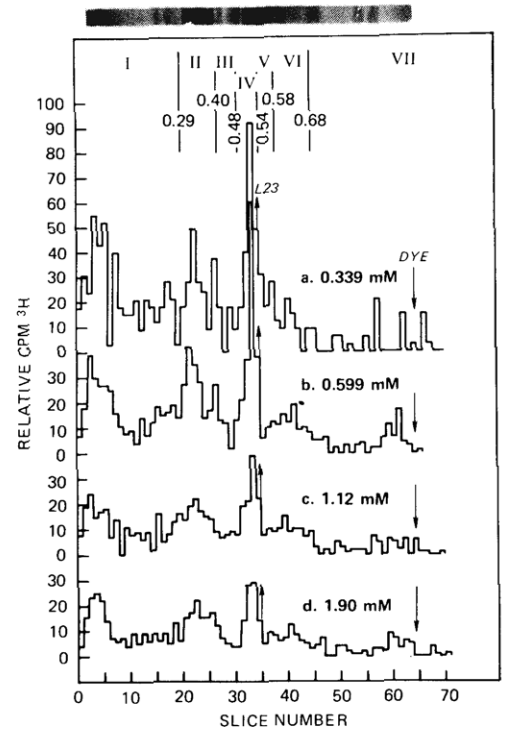


FIGURE 2: Polyacrylamide gel pattern of labeled proteins from 50S particles as a function of puromycin concentration. A stained gel is shown above the graph. The arrow pointing up marks the position of authentic L23. Experimental conditions: 111 $A_{260}$ units/mL K ribosomes; photolysis was for 8 min at 2537 Å. The specific activities of puromycin were: (a) 890 Ci/mol; (b) 504 Ci/mol; (c) 269 Ci/mol; (d) 159 Ci/mol. Reported counts per minute are for protein from 3.5 $A_{260}$ units of 50S particles.

Table I summarizes results obtained in one-dimensional gel electrophoretic analysis of labeled 50S and 70S proteins as the conditions of the labeling reaction are varied. The values listed inherently underestimate the specificity of region IV labeling, comparing as they do labeling of a narrow region (0.06 $R_f$ unit) to labeling of wider regions (0.11–0.12 $R_f$ unit) but the trends in the values are quite significant. Thus, labeling is clearly more specific for region IV at low rather than at high light fluences (no.'s 1–3, 11, and 18) and with T rather than P, D, or K ribosomes (no.'s 2, 4, 5, 7, and 8), but appears rather insensitive to the lamp used (no.'s 2 and 9, 6 and 10). The specificity of labeling decreases at low KCl concentration (no.'s 12–15). Addition of the structural and functional analogues of puromycin, CAPhe (no.'s 2, 16, and 17) and PhePANS (no.'s 18, 19, and Figure 8), reduces both the extent and specificity of region IV labeling. The labeling in region VI, which contains S14, is also reduced, although to a relatively lesser extent, while region II labeling is hardly affected. In a control experiment added dinucleoside phosphate CpA, which lacks the vital hydrophobic amino acid residue necessary for correct binding as a peptidyl acceptor (Nathans and Neidle, 1963; Harris et al., 1971), was shown to have no effect on the distribution of radioactivity in the one-dimensional gels.

*Overall Puromycin Incorporation.* The distribution of incorporated puromycin among the protein and RNA fractions of both 30S and 50S ribosomes (Cooperman et al., 1975) was systematically studied as a function of puromycin concentration (0.07–2 mM), time and wavelength (2537- and 3500-Å

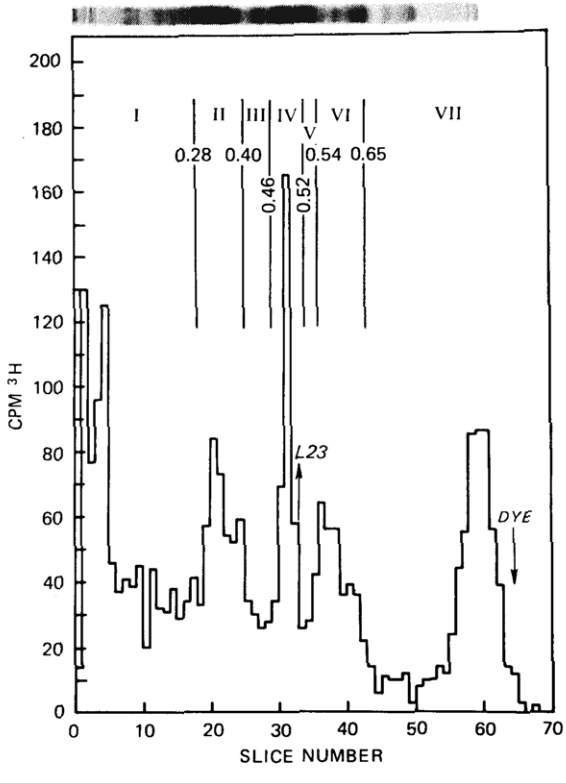


FIGURE 3: Polyacrylamide gel pattern of labeled proteins from 70S particles. A stained gel is shown above the graph. The arrow pointing up marks the position of authentic L23. Experimental conditions: 111 $A_{260}$ units/mL K ribosomes; 0.074 mM puromycin (2013 Ci/mol); photolysis was for 8 min at 2537 Å. Reported counts per minute are for protein from 5.6 $A_{260}$ units of 70S particles.

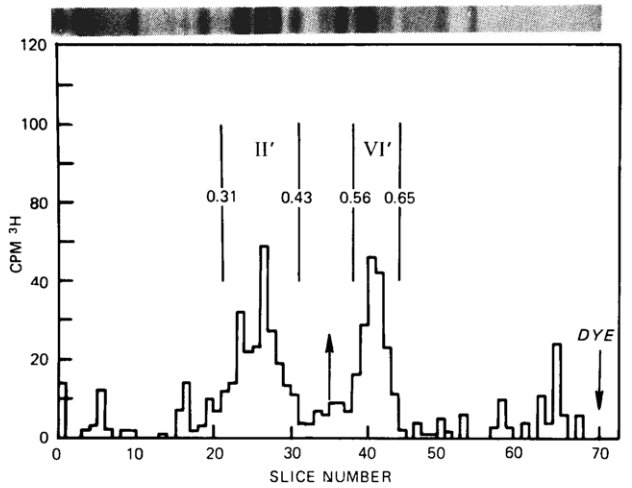


FIGURE 4: Polyacrylamide gel pattern of labeled proteins from 30S particles. A stained gel is shown above the graph. The arrow pointing up marks the position of the major radioactive peak in Figures 2 and 3. Experimental conditions: 111 $A_{260}$ units/mL K ribosomes; 0.339 mM puromycin (890 Ci/mol); photolysis was for 8 min at 2537 Å. Reported counts per minute are for protein from 1.15 $A_{260}$ units of 30S particles.

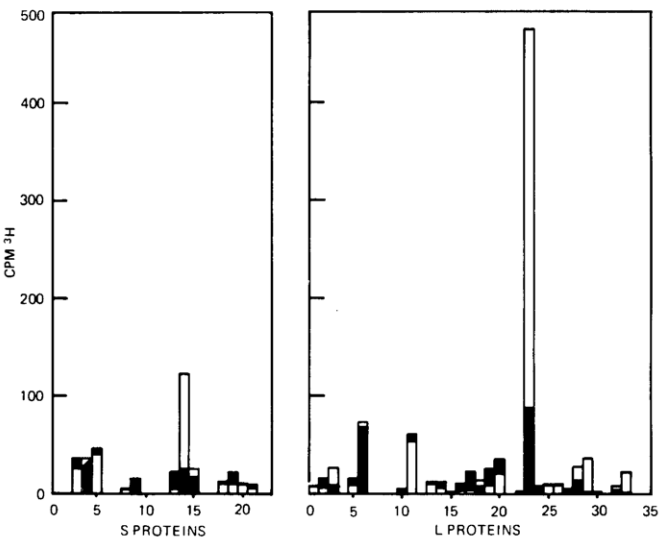


FIGURE 5: Two-dimensional gel electrophoresis pattern of ribosomal protein labeling at high and low puromycin concentration. Sample was applied to the anodic end in the first dimension, as in Figure 1c. The region surrounding the major radioactive peak, which includes proteins L18, L22, L23, and L25, was cut very finely, as in Figure 1c. The values given for L23 include the major radioactive peak centered at its upper left (see text). For all other proteins, large areas around each protein were cut out, as in Figure 1a. Counts reported are corrected for total recovery of radioactivity from the two-dimensional gels. Experimental conditions: 102 $A_{260}$ units/mL T ribosomes; photolysis was for 30 min at 3500 Å; (□) 0.1 mM puromycin, sp act. 1.68 Ci/mmol; (■) 2.0 mM puromycin, sp act. 0.08 Ci/mmol. In displaying the data the lower radioactivity value is superimposed on the higher one.

lamps) of irradiation, ribosomal preparation (K, T, and P), and KCl (25–205 mM) concentrations. For the most part only minor differences were noted. The labeling of 50S protein was found highest for T ribosomes where it amounted to 40% of total incorporation. A plot of the incorporation of puromycin into the RNA fraction of 50S subunits as a function of puromycin concentration is apparently biphasic, whereas the corresponding plot for 30S subunits yields a straight line (Figure 9).

Studies on the effects of puromycin analogues on incorporation are summarized in Table II. PhePANS blocks puromycin incorporation into 70S ribosomes to the extent of 35–40% at saturating levels and in radioactive form is itself incorporated to almost the same extent as puromycin. The inability of PANS to block puromycin incorporation demonstrates the specificity of the effect for PhePANS. CAPhe is also seen to partially block incorporation into ribosomal RNA, to the extent of about 30% at saturating values.

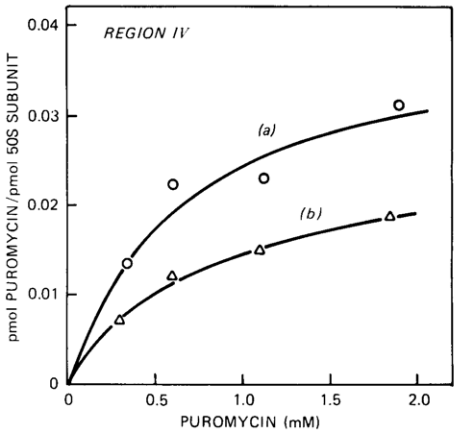


FIGURE 6: Saturation of region IV labeling with increasing puromycin concentration. Data obtained from two different experiments of the type shown in Figure 2. The solid lines are drawn from a least-squares fit of the data to a saturation function.

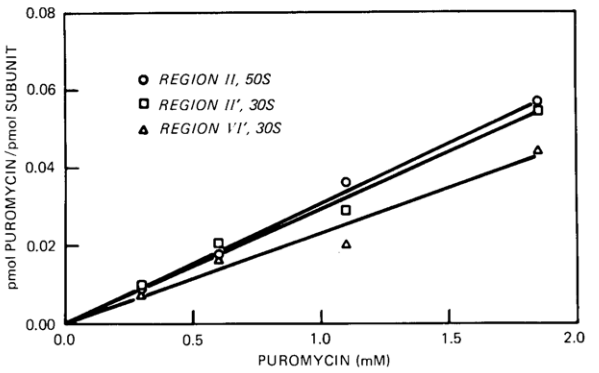


FIGURE 7: Incorporation of puromycin into one-dimensional gel regions as a function of puromycin concentration. Experimental conditions are described in Figure 2.

Addition of saturating amounts of 2-mercaptoethanol (>3 mM) to the photolysis mixtures inhibited incorporation into 70S ribosomes to the extent of 80%.[4] Similar results were obtained with dithiothreitol but inhibition with 2-aminoethanol was much less marked. In the presence of 3 mM dithiothreitol, a plot of puromycin incorporation vs. puromycin concentration gave a straight line, the curvature due to the presence of a saturable component seen in the absence of dithiothreitol (Cooperman et al., 1975) having disappeared. One-dimensional gel electrophoresis of proteins extracted from ribosomes labeled at saturating thiol concentration showed no discernible peaks of radioactivity.

*Acceptor Ability of Incorporated Puromycin.* Two attempts were made to test the acceptor ability of incorporated puromycin toward acylaminoacyl transfer as described in the Experimental Section. In the first, a combination of short photolysis time (3500-Å lamp) and low puromycin concentration was employed in obtaining ribosomes having a rela-

[4] By contrast, at saturating thiol concentrations only 40%, presumably all carbene independent, of the *N*-ethyl-2-diazomalonylpuromycin incorporation (Cooperman et al., 1975) was blocked.

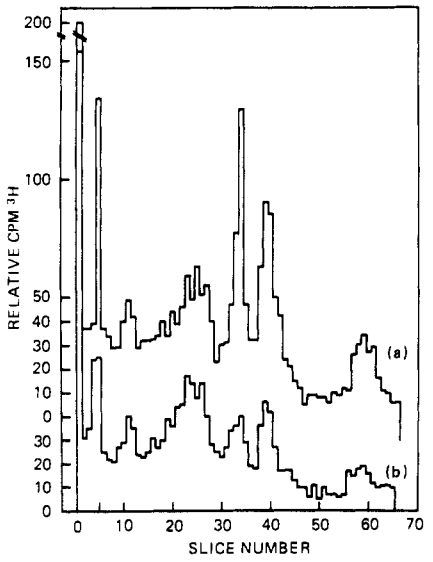


FIGURE 8: Effect of PhePANS on puromycin incorporation into total 70S ribosomal proteins. Experimental conditions: (a) 99 $A_{260}$ units/mL ribosomes; 0.15 mM puromycin (926 Ci/mol). Photolysis was for 120 min at 350 Å. (b) Same as (a), but with 2.1 mM PhePANS added. Reported counts per minute are per 1.4 $A_{260}$ units of 70S protein applied to gel.

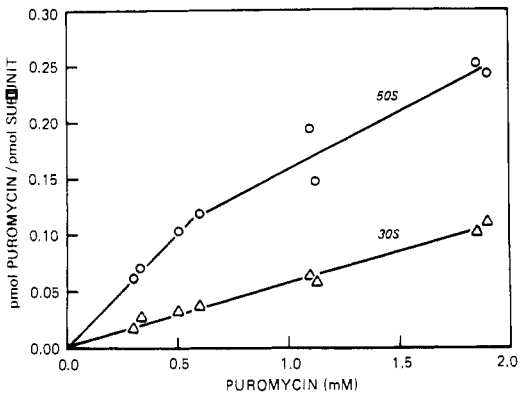


FIGURE 9: Concentration dependence of puromycin incorporation into 30S and 50S RNA. Solid lines are drawn through the average of two determinations at each puromycin concentration. Experimental conditions: 100 $A_{260}$ units/mL K ribosomes. Photolysis was for 8 min at 2537 Å. Proteins extracted from the 50S and 30S particles were used in Figures 2 and 4.

tively low level of puromycin incorporation (0.06/ribosome) but retaining virtually full activity in assays of both AcPhe-tRNA binding and of AcPhe transfer to noncovalently bound puromycin. In the second, a longer photolysis time and higher puromycin concentration resulted in a ribosome with a high level of puromycin incorporation (~3/ribosome) but retaining only partial activity (~50%) in both assays. In neither case was AcPhe transferred to covalently bound puromycin at levels appreciably above background.

## Discussion

Labeling of the major radioactive peak in the two-dimensional gels satisfies the important criterion for an affinity labeling process that it is blocked by excess puromycin. Similarly, labeling of region IV in one-dimensional gels reaches a saturation value as a function of puromycin (Figure 6) and is blocked by two puromycin analogues, CAPhe and PhePANS. The apparent $K_D$ for puromycin labeling calculated from these results, 0.7 ± 0.2 mM, is identical within experimental error with the value of 0.65 ± 0.26 mM previously obtained for the

TABLE II: Photoincorporation of Puromycin and PhePANS; Effect of Added Ligands.[a]

| Radioact. ligand | Added ligand, concn (mM) | Rel radioact. ligand incorp./ 70S particle | Rel radioact. ligand incorp./ total 70S RNA |
|---|---|---|---|
| [$CH_3$-$^3$H]Puromycin | | 1.00 ± 0.05 | |
| | PhePANS, 0.29 | 0.88 ± 0.04 | |
| | PhePANS, 0.59 | 0.92 ± 0.04 | |
| | PhePANS, 1.0 | 0.58 ± 0.05 | |
| | PhePANS, 2.1 | 0.68 ± 0.02 | |
| | PANS, 1.0 | 1.00 ± 0.15 | |
| | | | 1.00 ± 0.07 |
| | CAPhe, 0.0053 | | 0.86 |
| | CAPhe, 0.010 | | 0.82 |
| | CAPhe, 0.013 | | 0.82 |
| | CAPhe, 0.026 | | 0.74 ± 0.05 |
| | CAPhe, 0.052 | | 0.72 ± 0.13 |
| | CAPhe, 0.104 | | 0.68 ± 0.10 |
| | CAPhe, 0.207 | | 0.74 ± 0.12 |
| [8-$^3$H]Puromycin | | 0.94 ± 0.05[b] | |
| [8-$^3$H]PhePANS | | 0.66 ± 0.04[b] | |

[a] In all competition experiments, puromycin concentration was ≤0.1 mM. Experiments with CAPhe were performed with T ribosomes, a 3500-Å lamp, and 30–60-min irradiation. All other experiments were performed with K ribosomes, a 2537-Å lamp, and 8-min irradiation.[7] [b] Relative to [$CH_3$-$^3$H]puromycin incorporation under identical conditions.

saturable portion of the overall ribosomal labeling (Cooperman et al., 1975). This shows that region IV is labeled in the saturable process. Furthermore, these $K_D$ values are similar in magnitude to $K_m$ values for puromycin in the peptidylpuromycin and fragment assays (about 0.3 mM) (Fahnestock et al., 1970; Harris and Pestka, 1973).

We have used the results of one-dimensional gel analysis to obtain a $K_D$ value for puromycin in preference to the results for two-dimensional gels because of the greater inherent reproducibility of the former method. One potential problem with this approach is that region IV for 50S proteins contains proteins other than L23. Our one-dimensional gel is identical with that used by Mora et al. (1971) and these authors have located most of the L proteins on this gel. From their work region IV contains, in addition to L23, L13, L14, L15, L16, L17, and L18. We can use our two-dimensional gel data on labeled 70S proteins (Figure 1) to compare the amount of radioactivity found in the major radioactive peak (modified L23, vide infra) with that found in the six other proteins listed above. When this is done 80–90% of the radioactivity in region IV is seen to arise from modified L23. The fact that the background radioactivity due to incorporation into other proteins is so low compared to incorporation into L23 explains the straightforward saturation behavior seen in Figure 6. The two-dimensional gel results presented in Figure 5 provide evidence that S14 is also a site for affinity labeling, albeit a secondary one. Here the one-dimensional gel approach cannot be used because the radioactivity incorporated into S14 is too low to dominate region VI′ in the 30S protein gel (Figure 4).

Our gel results provide strong evidence that the major radioactive peak we see represents modified L23. The arguments are as follows: First, radioactivity in region IV arises from a 50S protein. The 50S proteins closest to the major radioactive spot in the two-dimensional gels are L23 and L18, and somewhat further away are L22 and L25. The simplest assumption

about a modification is that it effects migration in a qualitatively similar manner in both electrophoretic dimensions. If so, we should expect radioactivity to comigrate with the native protein, to migrate to the upper left (retarded in both directions) of native protein, or to migrate to the lower right (accelerated in both directions) of native protein. The major radioactive peak lies to the upper left of L23, but to the lower left of L18, the upper right of L25, and the direct left of L22. Secondly, from the work of Mora et al. (1971), L22 and L25 migrate considerably faster on the one-dimensional gel than the major radioactivity peak (4 gel slices or 0.06 $R_f$ unit) and can be excluded on this basis. L18 comigrates with L23, i.e., slightly faster than the major peak of radioactivity. The one-dimensional gel is run at pH 4.5, similar to the pH (4.6) of the second dimension in the two-dimensional gel. Yet the major peak of radioactivity on the two-dimensional gel actually migrates faster than L18 in the second dimension. As it is unreasonable to expect that the change in acrylamide concentration between the two gels would change the relative electrophoretic mobilities of modified compared with native protein, these results make it unlikely that L18 is the incorporation site. L23, which migrates faster than the major radioactive peak under both conditions, again emerges as the most likely incorporation target. This conclusion has recently been confirmed by immunoprecipitation experiments, showing that anti-L-23 precipitates a major fraction (~40%) of the total radioactivity incorporated into 50S protein. By contrast, none of the other proteins in the vicinity of the major radioactivity peak in the two-dimensional gel (L18, L19, L21, L22, and L25) precipitates more than 5% of this radioactivity.[5]

The two-dimensional gel results are more definitive by themselves in identifying S14 as the second most important protein labeled by puromycin. In this case not only does the radioactivity comigrate with S14, but also S14 is very well resolved in the Kaltschmidt and Wittmann system (especially when run as in Figure 1c) from all other ribosomal proteins (Kaltschmidt and Wittmann, 1970). The immunoprecipitation results are again in accord with this identification. Anti-S14 precipitates 20–25% of the total radioactivity in 30S protein, which is more than twice as much as that found with antibodies to any of the 30S proteins anywhere near S14 on the two-dimensional gel (S12, S13, S18, S19).[5]

L23 and S14 are probably not the only ribosomal components specifically labeled from the puromycin binding site. Our earlier data (Cooperman et al., 1975) indicated that at puromycin concentrations low with respect to $K_D$ (i.e., below 0.2 mM) approximately 50% of the observed puromycin incorporation occurs via a puromycin saturable site (i.e., via affinity labeling). This is consistent with our finding that the puromycin analogue PhePANS blocks approximately 40% of puromycin incorporation at saturating concentrations (Table II). Our best estimate from our gel results, which agree very well with the recent immunoprecipitation results cited above, is that 14% of the total incorporated puromycin is in L23 and an additional 4–5% is in S14. There remains therefore approximately 25% of the incorporation which proceeds via puromycin-specific binding into ribosomal sites which have not as yet been identified. The results that CAPhe at saturating concentration shows approximately 30% inhibition of total RNA labeling (Table II), and that incorporation into 50S RNA (but not 30S RNA) shows curvature as a function of puromycin concentration (Figure 9), suggest that a portion of 50S RNA provides one of these sites, accounting for 10–15% of the overall incorporation.[6] Studies to localize the putative labeled RNA region are now in progress. Here it should be noted that affinity labeling results obtained with an electrophilic analogue of puromycin substituted at the 5′ position indicate 23S RNA to be the site of affinity labeling (Greenwell et al., 1974) although this result should be considered as tentative given the very high degree of nonspecific labeling found in this study. It is likely that the remaining 10–15% of incorporation arising from a site specific process is spread in small amounts over other ribosomal proteins which our current methods of analysis would be inadequate to identify with confidence.

CAPhe and PhePANS partially block overall incorporation into either ribosomes or ribosomal RNA. The data presented in Table II allow calculation of approximate $K_D$ values of 1 mM for PhePANS and 0.01 mM for CAPhe. The ratios $K_D^{puromycin}/K_D^{PhePANS}$, equal to 0.7, and $K_D^{puromycin}/K_D^{CAPhe}$, equal to 70, are similar to the corresponding $K_m$ ratios (0.7 and 37, respectively) previously found in peptidyltransferase assays (Harris et al., 1971; Ringer and Chladek, 1974), providing evidence that inhibition of labeling is occurring at the binding site for the 3′ terminus of tRNA within the functional peptidyl acceptor, or A, site.

Puromycin is an analogue of the 3′ terminus of aminoacyl-tRNA which, given the peptidyltransferase reaction, must be bound on the ribosome close to the 3′ terminus of peptidyl- or acylaminoacyl-tRNA. In this context, assignment of L23 as forming part of the puromycin binding site agrees with results of other studies of ribosome topology. Thus, immune electron microscopy studies (Tischendorf et al., 1975) have shown L23 to be close to L11 and L18, the two proteins which are the principal incorporation sites for two photolabile *N*-acyl-Phe-tRNA$^{Phe}$ derivatives (Hsiung and Cantor, 1974; Hsiung et al., 1974) and to L16, the apparent site of chloramphenicol binding (Pongs et al., 1973, 1974). In addition, L23 binds directly to a region of 23S RNA which includes the incorporation sites of two other reactive *N*-acyl-Phe-tRNA$^{Phe}$ derivatives, one photolabile (Sonenberg et al., 1975) and one electrophilic (Yukioka et al., 1977).

A variety of results, as discussed by Stöffler (1974) and Cooperman (1977), have led to the conclusion that the peptidyltransferase locus of the ribosome overlaps with at least part of the 30S·50S interface. Our finding that, in addition to L23, S14 appears to be affinity labeled suggests that these two proteins may be close to one another in the intact ribosome, and it is interesting that both of these proteins have been shown to be at the interface. Additional support for this view comes from recent work in this laboratory[5] showing that in the presence of chloramphenicol S14 becomes the major site of puromycin incorporation.

Several of the problems common to affinity labeling studies on ribosomes (Cooperman, 1977) are confronted directly in the experiments summarized in Table I and Figures 6 and 7. Thus L23 is labeled more specifically at low rather than high radiation fluence (Table I, no.'s 1–3), from which it may be concluded that labeling of L23 occurs with the native ribosome structure.[7] L23 is also labeled more specifically at low rather than high puromycin concentration (Figures 6 and 7) showing

[5] P. G. Grant, E. N. Jaynes, Jr., W. A. Strycharz, B. S. Cooperman, and M. Nomura, manuscript in preparation.

[6] This curvature cannot be due to a change in the principal photoexcited species in solution as puromycin concentration is increased, since the maximum contribution of puromycin absorbance to total absorbance of the UV- (2537 Å) irradiated solutions was only of the order of 10–15%, and even this small increase was corrected for as previously described (Cooperman et al., 1975) in obtaining the data presented in Figure 9.

[7] The half-time for loss of poly(U)-dependent polyphenylalanine synthesis activity under the conditions of the incorporation experiment is 10 min (Cooperman et al., 1975).

labeling to occur from a specific puromycin binding site. L23 is found to be the major labeled protein for ribosomes prepared by a variety of procedures (Table I, no.'s 2, 4–7), including one which employs high salt washing (K ribosomes) and one which does not (T ribosomes). Here it is worth noting that L23 is one of the 50S proteins tightly bound to 23S RNA and not easily removable by salt washing (Hindennach et al., 1971). Finally, L23 remains the major labeled protein as KCl concentration is varied (Table I. no.'s 12–15). These results suggest that L23 is a highly conserved part of the puromycin binding site.

Two uncertainties remain with respect to placing L23 at the puromycin binding site. First, it is possible that L23 is simply a protein close to the site which has a high reactivity toward puromycin under the conditions of irradiation [although L23 is not one of the more photolabile 50S proteins (Gorelic, 1976)]. Secondly, we were unable to detect AcPhe transfer to incorporated puromycin. This may indicate that incorporation is not, even partly, at a functional site. However, alternative explanations, for instance that puromycin incorporation occurs at a vital catalytic center for peptidyltransferase, thus inhibiting this activity, or that the photochemical reaction leading to incorporation modifies the puromycin molecule in such a way that it is no longer a competent peptidyl receptor, are also possible. Work with photolabile derivatives of puromycin with the potential to solve both of these problems is currently underway.

At a concentration of puromycin (0.3 mM) at which 30% of the ribosomal puromycin sites are filled ($K_D$ = 0.7 mM), irradiation at 2537 Å with a light dose such that each puromycin in solution absorbs 1.2 quanta results in the incorporation of 0.18 puromycin molecule per ribosome, with half of the incorporation being site specific and half nonspecific (Cooperman et al., 1975). Assuming that incorporation results from reaction of a photoexcited puromycin, these results lead to calculated quantum yields of 0.25 for the affinity labeling reaction, and of 0.001 for nonspecific labeling. The quantum yield for photodestruction of adenine in nondeoxygenated aqueous solution on 2537-Å irradiation is known ($\sim 10^{-4}$) (Ivanchenko et al., 1975; Shugar, 1960). In experiments not shown, we have found by measuring the loss of purine absorption that photodestruction of the the *N,N*-dimethyladenine moiety of puromycin on irradiation at 2537 Å proceeds with a quantum yield approximately 6 times that for the photodestruction of adenosine. Thus, the quantum yield for the aqueous photodestruction of puromycin is very similar to that calculated for the nonspecific incorporation reaction, so that it is at least plausible that photodestruction leads to nonspecific incorporation. The much higher calculated quantum yield for the affinity labeling reaction suggests that here a different process is involved. One possibility is the 8-alkylation of the purine moiety by either an amine or an alcohol, a process which has been observed for several adenine-containing molecules (Salomon and Elad, 1974; Leonov et al., 1973) and which we have found to be true for puromycin as well.[8] This possibility can be excluded, for not only is total incorporation with [8-$^3$H]puromycin found to be about the same as that with [$CH_3$-$^3$H]puromycin (Table II), but also polyacrylamide gel electrophoretic labeling patterns obtained using both kinds of isotopically labeled puromycin are essentially identical. [8-$^3$H]PhePANS is incorporated into ribosomes about 70% as well as puromycin itself (Table II) providing some evidence against the involvement of the *O*-methyltyrosine moiety (Bent and Hayon, 1975). Other possibilities would be that the large apparent quantum yield results from internal energy transfer from electrically excited ribosomal components to bound puromycin, or that excited ribosomal components insert into puromycin rather than vice versa.

[8] D. E. Calderon, E. N. Jaynes, Jr., and B. S. Cooperman, unpublished observations.

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# Regulation of Translation Rate during Morphogenesis in the Fungus *Mucor*†

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ABSTRACT: The present study was undertaken in order to elucidate the molecular mechanisms responsible for regulating changes in the specific rate of protein synthesis during the yeast-to-hyphae morphogenesis in the fungus, *Mucor racemosus.* The distribution of ribosomes between active polysomes and monosomes and inactive subunits was determined by means of pulse-labeling and density gradient fractionation techniques. The percentage of ribosomes active in protein synthesis was observed to decrease throughout the morphological transition. The rate of amino acid addition to nascent polypeptide chains was calculated and the transit time of messenger RNA translation was measured. The results showed a significant increase in the velocity of ribosome movement along the message which was continuously adjusted throughout hyphal development.

*Mucor racemosus* is a dimorphic phycomycete which can be stimulated to undergo a rapid and synchronous morphological change by altering the composition of the atmosphere under which the cells are grown. The cells grow as budding yeasts under a $CO_2$ atmosphere in the presence of a hexose. Changing the atmosphere from $CO_2$ to air results in the emergence of hyphae from the yeast cells (Bartnicki-Garcia and Nickerson, 1962; Larsen and Sypherd, 1974). Measurements of the kinetics of radioactive amino acid incorporation into protein indicate that there is an acceleration in the specific rate of protein synthesis during initial germ tube emergence which later declines during further hyphal elongation (Orlowski and Sypherd, 1977). Protein is synthesized throughout

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